IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: P/4639-2

In re Application of:	)	Art Unit: 1654
	)	
Ilana Nathan et al.	)	Examiner: Duffy, Bradley
	)	
Appln. No.: 10/509,405	)	Washington, D.C.
	)	
Date Filed: September 24, 2004	)	Confirmation No. 5690
	)	
For: Compositions for Treating and Preventing Necrosis	)	

DECLARATION OF Ilana Nathan UNDER 37 C.F.R. § 1.132

Sir:

I, the undersigned Ilana Nathan, hereby declare and state as follows:

1. I am a researcher at the Ben-Gurion University of the Negev, Be'er Sheva, Israel. My research focus includes work on cell necrosis and apoptosis, and my understanding and experience on these topics is extensive.

2. Certain experimentation related to the invention described in the above-identified application has been conducted in my laboratory at the Ben-Gurion University of the Negev. This experimentation is described in the manuscript attached hereto as Exhibit 1.

3. I either conducted or supervised all of the experimentation described in the manuscript of Exhibit 1. I hereby state, from my own first-hand knowledge, that all of the

statements therein are true and the results described therein are true and accurate.

4. The experiments described in Exhibit 1 further support the finding that z-VAD-fluoromethylketone (z-VAD-fmk), in combination with elastase inhibitors, such as elastase inhibitor III (MeOSuc-Ala-Ala-Pro-Val-CMK), effectively serve to reduce KCN-induced cell necrosis.

5. Based on my knowledge and experience, it is also my belief and understanding that one of ordinary skill in the art at the time of filing of the present application, in 2004, would recognize and understand that z-VAD-fluoromethylketone (z-VAD-fmk) to be a typical member species among the class of effective anti-apoptotic agents known as caspase inhibitors.

I hereby further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

23/12/08
Date

I. Nathan
Ilana Nathan

U.S. Pat. App. 10/509,405
Declaration of Ilana Nathan
Exhibit 1 – z-VAD-fmk + elastase inhibitor III STUDIES REPORT

Background.
The tri- peptide sequence "VAD" is broadly recognized by all group 1 caspases and z-VAD- fluoromethylketone (fmk) is a powerful, irreversible and cell permeable inhibitor for caspases which are known to play a key role in apoptotic cell death program.

Methods
The U-937 cells are a p53 minus monocytic cell line (ATCC: CRL 1593). The cells were grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 Mm glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell culture products were purchased from Beit Haemek, Israel. The cells were split every third day, ensuring that the cells grow in logarithmic phase.
U-937 cells cultured in complete RPMI-1640 medium were washed and seeded in glucose-free RPMI-1640 medium as described below and treated with or without 5 or 10 mM KCN (Merck, Germany) for seven hours.

Cell viability was determined by the trypan blue exclusion method. In addition, cells undergoing morphological changes associated with apoptotic or necrotic cell death were monitored. At the given time point after treatment, 900 μl of the medium was collected, centrifuged, the pellet was resuspended, stained with dye mixture (composed of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) and placed on a glass slide to be viewed in a fluorescence microscope. Cells were scored as alive if their nuclei exhibited normal morphology and were green. Cells exhibiting normal morphology and orange color were indicated as necrotic. Cells were scored as apoptotic if their nuclei exhibited condensation of the chromatin and/or nuclear fragmentation. A minimum of 100 cells was scored for each sample.

The role of caspases in apoptotic cell death pathway is well established. The pan-caspase inhibitor z-VAD-fmk was used at low concentration together with relatively low concentration of elastase inhibitor III to block KCN induced necrotic cell death induced in U937 cells. 10 or 20 μM of z-VAD-fmk (Alexis biochemicals) and/or 100 μM of elastase inhibitor III (Calbiochem) were added to U 937 cells 30 minutes before the cells were treated with KCN to allow the inhibitors to enter the cell. During an hour before the addition of the inhibitors the cells were maintained in glucose free medium to allow adaptation to the new environment. 7 hours later cell survival was analyzed by ethydium bromide and acridine orange double staining and trypan blue exclusion.

Results
The following experiments were carried out on the basis of our previous results showing that elastase inhibitor III at relatively low concentrations, was able to shift necrotic cell death to apoptotic cell death. We used a combination of elastase inhibitor III, at concentration in which it is only slightly active by itself in the experimental system, together with relatively low concentrations of z-VAD-fmk. The effect of the combination of both compounds in inhibition of necrotic cell death induced by KCN in U937 cells was studied and compared to the effect of the individual compounds. The effect on necrosis was assessed by different methods.

Fig. 1 describes the effect of the combination of elastase inhibitor at a concentration of 100 μM and z-VAD-fmk at a concentration of 20 μM on necrosis as measured by determination of cell viability. It can be seen that both inhibitors were not cytotoxic by themselves. KCN caused dose dependent cell death as manifested by decrease in cell survival. Both inhibitors were not able to protect the cells from KCN induced cell death by themselves. However, a significant effect in prevention of the decrease in cell survival induced by KCN was observed when both compounds were co administered.

A significant effect of the co administration of z-VAD-fmk with elastase inhibitor III is also apparent in the prevention of KCN induced cell death as measured by determination of percent of necrotic cells (**Fig. 2**). KCN caused dose dependent increase in percent of necrotic cells. Elastase inhibitor at a concentration of 100 μM and z-VAD-fmk at a concentration of 20 μM are unable to protect the cells from necrosis when each one is used separately. In addition, neither compound was toxic when used alone or in combination. However, these two inhibitors together showed a significant effect in prevention of necrosis. Under these conditions the mean percent of apoptotic cells did not exceed 7.75. Similar behavior of protection from necrotic cell death by the combination of the two inhibitors was observed measuring LDH release for assessment of necrotic cell death (data not shown). These results indicate the benefit in using a combination of elastase inhibitor together with antiapoptotic agent to prevent necrotic cell death.

Summary

The combined action of elastase inhibitor III and z-VAD-fmk has a significant effect in the prevention of necrosis.

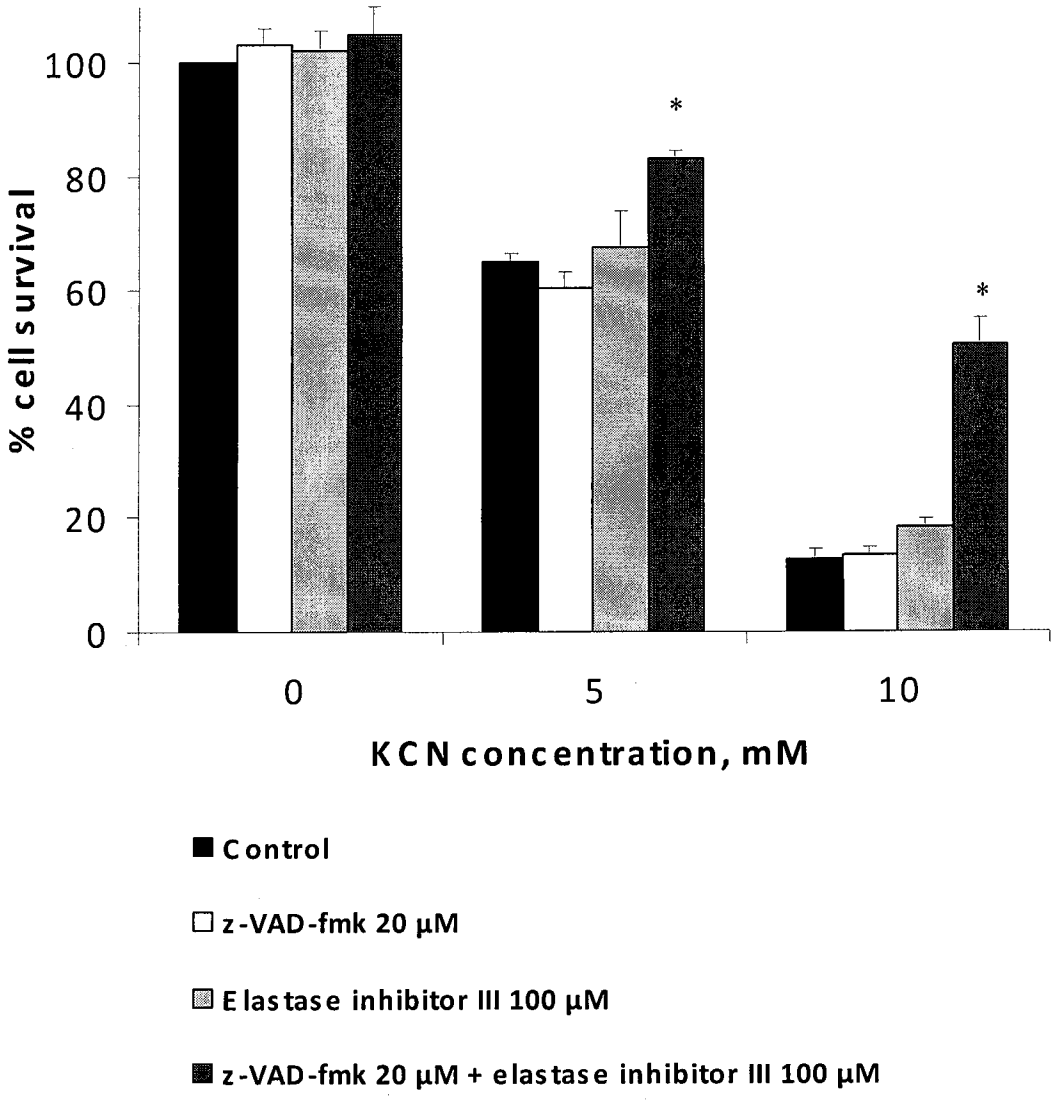

Figure 1. The protective effect of a combination of z-VAD-fmk with elastase inhibitor III on KCN-induced necrosis in U-937 cells as assessed by trypan blue exclusion. Cells were maintained in glucose-free medium, pre-incubated with z-VAD-fmk or with elastase inhibitor III or with a combination of these two compounds for 30 min, and then KCN was added for seven hours. Thereafter alive cells were stained and counted by trypan blue exclusion.*$P < 0.02$.

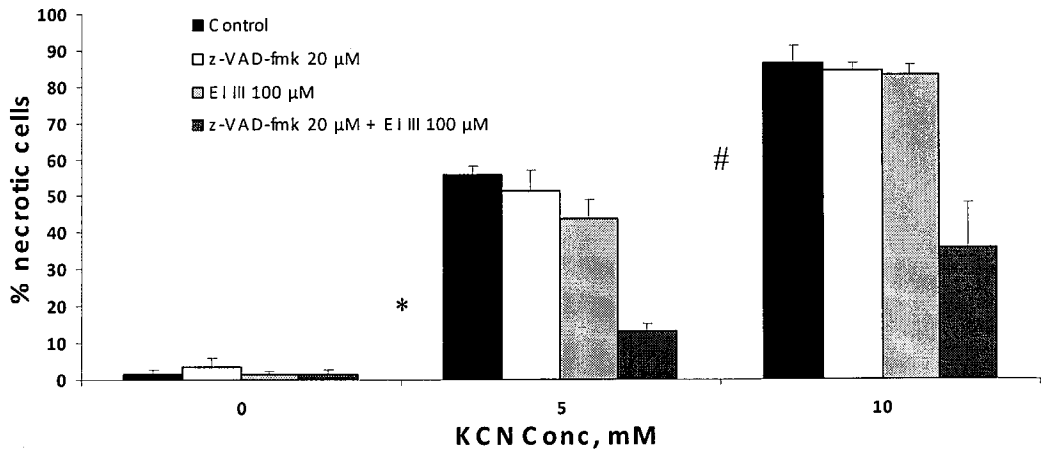

Figure 2. The effect of a combination of z-VAD-fmk with elastase inhibitor III on KCN-induced necrosis in U-937 cells as assessed by ethydium bromide and acridine orange double staining. Cells were maintained in glucose-free medium, pre-incubated with z-VAD-fmk or with elastase inhibitor III or with a combination of these two compounds for 30 min, and then KCN was added for seven hours. Thereafter necrotic cells were stained and counted by ethydium bromide and acridine orange double staining. *$P < 0.0004$, # $P < 0.003$